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Summary

Innate immunity is critical for shaping vaccine-elicited adaptive immune responses. Several classes of immune sensors, including Toll-like receptors, retinoic acid-inducible gene-I-like receptors, nucleotide-binding oligomerization domain-like receptors and cytosolic DNA receptors mediate important innate immune pathways and provide potential targets for novel adjuvant development. Understanding how innate immunity modulates adaptive immune responses will probably be important for optimizing vaccine candidates. Here, we review recent advances in innate immunity, focusing upon their potential applications in developing adjuvants and vectors for HIV vaccines.

Keywords: HIV, innate immunity, TLR, vaccines

Introduction

The development of an effective HIV vaccine remains a critically important yet elusive goal. Despite advances in preventing HIV transmission and treating chronic HIV infection, investigators have not been successful in developing a vaccine, reflecting the major scientific challenges in generating effective antibody and T lymphocyte responses to HIV [1,2]. At the same time, advances in the field of innate immunity have led to the realization that the innate immune system contributes significantly to the ability of vaccines to generate adaptive immune responses against pathogens [3–5]. Moreover, licensed vaccines and adjuvants have been shown to activate innate immune signalling pathways. For example, the live-attenuated yellow fever vaccine (YF-17D) activates multiple innate immune receptors [6,7], suggesting a model of innate immune activation upon which a protein-based or non-replicating vaccine platform could be patterned. Harnessing innate immunity therefore offers considerable promise in designing the next generation of HIV vaccine candidates [8].

The innate immune system is comprised of a network of different cell types, including dendritic cells (DCs), macrophages, natural killer (NK) cells, NK T cells and gamma–delta T cells that are poised to encounter pathogens in the first minutes to hours of infection. Unlike adaptive immunity, which is characterized by the narrow specificity of host–pathogen recognition and the ability to generate immune memory, the hallmark of innate immunity is its ability to recognize pathogen motifs and initiate the induction of rapid, first-line effector responses [4,9,10]. Innate immunity therefore encompasses numerous cell types and functions, of which a subset is dedicated to modulating adaptive immune responses. Early innate effector functions may include the secretion of proinflammatory cytokines and chemokines, secretion of anti-viral type I interferons (IFNs), induction of acute-phase reactants, activation of complement and recruitment of inflammatory cells [9–11]. The role of these innate immune defences in the pathogenesis of acute and chronic HIV infection remains poorly defined, but is an important area of ongoing investigation. Further insights may contribute to the development of immunomodulatory therapies that can augment early innate host defences against HIV infection [8,12–15].

Innate immune recognition is based upon pattern recognition receptors (PRRs) that are present on several types of innate immune cells and possess a broad specificity capable of detecting common structural motifs or pathogen-associated molecular patterns (PAMPs) that are present in bacteria, viruses, fungi and parasites [9,11,16]. Engagement

Table 1. Pattern recognition receptors (PRR) and adjuvants.

PRR family	Innate receptor	Natural ligands	Adjuvants	References
Toll-like receptors (TLR)	TLR-1 + TLR-2	Triacyl lipopeptides	Pam3Cys	[65]
	TLR-2 + TLR-6	Diacyl lipopeptides	MALP-2, Pam2Cys	[66,67]
	TLR-2	Peptidoglycans	BCG, CFA	[68,69]
	TLR-3	Double-stranded RNA	Poly I:C	[70]
	TLR-4	Lipopolysaccharide	MPL A, BCG	[43,71]
	TLR-5	Flagellin	Flagellin	[72]
	TLR-7, TLR-8	ssRNA	Imiquimod, Resiquimod, R848	[73]
	TLR-9	Unmethylated CpGs	CpG ODN	[74]
RIG-I-like receptors	RIG-I	Double-stranded RNA		[47]
	MDA-5	Double-stranded RNA	Poly I:C	[47,75]
NOD-like receptors	NOD1	Peptidoglycans	FK156, FK565	[76]
	NOD2	Muramyl dipeptide	CFA	[18,77]
	Nalp3	Bacterial RNA, Uric acid crystals	R837, R848, Alum	[44,78]
Cytosolic DNA sensor	DAI	Plasmid DNA		[46]

Pam3Cys, *N*-palmitoyl-(*s*)-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-cysteine; MALP-2, macrophage activating lipopeptide-2; Pam2Cys, *S*-[2,3 bis(palmitoyloxy)propyl]-cysteine; BCG, bacille Calmette–Guérin; CFA, complete Freund's adjuvant; poly I:C, polyriboinosinic-polyribocytidilic acid; MPL A, monophosphoryl lipid A; CpG, cytosine-guanine dinucleotide; ODN, oligodeoxynucleotide; NOD, nucleotide-binding oligomerization domain 2; DAI, dopamine-1 receptor agonist; RIG, retinoic acid-inducible gene.

of PRRs results in activation of several innate host defence functions and initiation of antigen-specific adaptive immunity. Several classes of PRRs have been discovered, including Toll-like receptors (TLRs), which have been the most studied in the setting of vaccine applications [3,4]. Moreover, an expanding array of non-TLR PRRs have been identified, including retinoic acid-inducible gene (RIG)-I-like receptors, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and cytosolic DNA receptors, which are also capable of triggering innate immune responses [10,17,18]. Here, we review recent advances in innate immunity, focusing upon their potential applications in developing adjuvants and vectors for HIV vaccines.

The TLR ligands

The best-studied class of PRRs are the TLRs, with up to 15 types identified in mammals [3]. Because of their diversity and ability to stimulate innate immunity and facilitate adaptive immune responses, several TLR ligands have been studied as potential vaccine adjuvants [5,19,20]. Recently, a TLR-4 agonist, monophosphoryl lipid A, was licensed in Europe for use in a hepatitis B vaccine [21], and other TLR ligands have appeared promising in clinical trials [20,22].

The TLRs are transmembrane receptors that recognize several PAMPs (see Table 1), including unique bacterial components such as lipoproteins (recognized by TLR-1 and TLR-2), lipopolysaccharide (LPS) (recognized by TLR-4) and flagellin (recognized by TLR-5). TLRs also recognize viral nucleic acid motifs, including double-stranded RNA (recognized by TLR-3) and single-stranded RNA (recognized by TLR-7, TLR-8). Unmethylated cytosine-guanine dinucleotide (CpG) DNA sequences found in viruses and bacteria are the natural ligands for TLR-9. Once activated, TLRs recruit intracellular adaptor proteins [either myeloid differentiation primary response gene (88) (MyD88) or Toll/interleukin-1 receptor domain-containing adaptor-inducing IFN-β], and initiate a signalling cascade leading to secretion of proinflammatory cytokines and activation of antigen-presenting cells (APCs). The pattern of cytokine secretion and immune cell activation is determined by the set of TLRs that are triggered and the cells expressing these TLRs [16].

The TLRs are localized within different cell compartments and display distinct patterns of expression depending upon cell type [17,23]. TLRs 1, 2, 4, 5 and 6 are located on the cell surface and typically recognize ligands that are expressed on a pathogen's surface, such as LPS or flagellin. In contrast, TLRs 3, 7, 8 and 9 are located intracellularly in the endosome; their ligands, which are primarily viral nucleic acids, become available only after lysosomal degradation of pathogens or cells. These patterns of TLR localization highlight the potential importance of targeting antigen and adjuvant to the same compartment within APCs. For example, it has been shown that optimal vaccine formulations include direct conjugation of antigen and adjuvant, or incorporation of both antigen and adjuvant into a vehicle that enables entry into the same endosomal pathway [19,24]. Moreover, expression of TLRs is distinct among different cell types. TLR-2 and TLR-4, for example, are expressed on several types of innate immune cells including macrophages, myeloid DCs (mDC), B cells and endothelial cells. In contrast, TLR-2 and TLR-4 are not present on plasmacytoid DCs (pDCs), which instead express TLR-7 and TLR-9 [3]. The pattern of TLR expression has been shown to determine whether these cell types can be activated by cognate TLR adjuvants [25], and emerging evi-

dence suggests that the nature of the DC subset and TLRs triggered play critical roles in modulating the adaptive immune response [23].

The TLR modulation of infection

Augmenting early innate immune responses may thwart infection or limit the early replication and dissemination of HIV, which may in turn provide an improved environment for adaptive immune protection. Such an approach applies most directly to the development of topical microbicides and novel anti-viral therapies, but may also pertain to candidate HIV vaccines that activate PRRs. Activation of innate immunity with TLR ligands has been shown to reduce viral replication in models of herpes simplex virus, hepatitis C and influenza infection in animal studies [26–28], supporting the development of a similar approach for HIV. Strategies directed at augmenting early immune defences have also targeted anti-viral responses shown to potentially limit HIV infection. This may include eliciting greater expression of chemokines such as regulated upon activation normal T cell expressed and secreted, macrophage inflammatory protein (MIP)-1α and MIP-1β, which can restrict utilization of chemokine receptor 5 (CCR5) by HIV for cell entry [29]. The ability of TLR ligands to elicit type I IFN expression may provide another pathway to elicit anti-viral responses, as type I IFNs play a key role in activation of other innate immune cells such as NK cells [30] and can enhance the expression of anti-viral proteins that prevent HIV replication, such as apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G). In one study a TLR-3 agonist, polyriboinosinic-polyribocytidilic acid (poly I:C), induced expression of type I IFN-mediated up-regulation of APOBEC3G in DC cultures [31].

The ability of TLR or PRR ligands to generate effective early, non-specific immunity against simian immunodeficiency virus (SIV) or HIV challenge has yet to be proved in an animal model. No protection was observed in a study in which rhesus macaques were treated with either a topical TLR-7/TLR-8 or TLR-9 adjuvant at mucosal surfaces and challenged with SIV [32]. Moreover, prolonged activation of innate immunity may not be beneficial. Elevated serum levels of LPS [33], as well as increased activation of pDCs and IFN-α secretion [34,35], have been observed in HIV-infected patients, and such chronic TLR stimulation may contribute to immune dysregulation noted with chronic HIV infection. Future efforts in developing PRR ligands to augment innate immunity as a first-line defence will need to focus upon how to elicit effective early anti-HIV immune responses without evoking potentially harmful innate immunity.

The TLR ligands as HIV vaccine adjuvants

Currently, utilization of TLR-directed adjuvants in candidate HIV vaccines has been limited to studies in mice and non-human primates [24,36–40]. Recent experiments in non-human primates have shown that superior T helper type 1 (Th1) responses were elicited by an HIV Gag protein conjugated to a TLR-7/TLR-8 agonist when compared with an unconjugated vaccine formulation [24]. Furthermore, the conjugated HIV-Gag–TLR-7/TLR-8 vaccine was able to elicit Gag-specific $CD8^+$ T cell responses, possibly because of enhanced cross-presentation. These effects were not detected when HIV Gag was administered with CpG oligodeoxynucleotide or a free TLR-7/TLR-8 agonist [24]. In a subsequent study, the same investigators studied TLR adjuvants in a prime-boost vaccine regimen [38]. The priming immunization included HIV Gag and an emulsified TLR-7/TLR-8, TLR-8 or TLR-9 agonist followed by a boost with a viral vector expressing HIV Gag. Each adjuvant affected the magnitude and quality of the T cell responses seen prior to and after the boost, with the TLR-7/TLR-8 adjuvant eliciting the highest frequency of long-lived polyfunctional Th1 and $CD8^+$ T cells. Delivery of the antigen and adjuvant to the same compartment (by conjugation or emulsification), as well as activation of different DC subsets (as TLR-7 is expressed in pDC and TLR-8 in mDC), contributed to the enhanced adaptive immune responses elicited by these protein–adjuvant formulations [3,23].

The TLR adjuvants can also augment immune responses to plasmid DNA vaccines [36,40]. Kwissa *et al.* demonstrated that the addition of a TLR-9 ligand to a plasmid DNA expressing SIV proteins improved the magnitude and polyfunctionality of antigen-specific $CD8^+$ T cell responses in rhesus macaques [40]. Moreover, in this study DCs were targeted by Flt3-ligand administration prior to vaccination, expanding the number of DCs *in vivo*. Immunization with DNA-GagPol and a TLR-7/TLR-8 or TLR-9 ligand enhanced activation of these expanded APCs [40]. Other strategies to enhance targeting of DCs by TLR ligands have also been explored by other groups. For example, in one study, DEC-205-expressing DCs were targeted by conjugating a vaccine protein to an α-DEC-205 antibody, which resulted in enhanced efficiency of antigen presentation [41,42]. In this system, the addition of the TLR-3 agonist (poly I:C) to HIV Gag p24-DEC205 augmented long-lived, polyfunctional Th1 responses in mice, although there was no improvement in $CD8^+$ T cell responses [39].

Although TLR agonists have been shown to enhance cellular and humoral responses to proteins and DNA-plasmid encoded antigens [17,20,43], their ability to augment these responses to HIV or SIV antigens has remained relatively untested. Further studies addressing the immunogenicity of specific TLR ligands (including those to TLR-4 and TLR-5) should be pursued. Moreover, optimization of these adjuvants will probably also require improved methods for co-delivery of antigen and adjuvant, targeting to DCs and other APCs and determining the optimal combination of TLR and non-TLR PRR activation.

Non-TLR innate immune receptors

Over the last few years several new classes of PRRs have been identified, providing potential novel targets for vaccine adjuvants (see Table 1). These receptors are present in the cytoplasm and may provide redundant signalling pathways for adjuvants or TLR ligands. Their existence explains why several potent adjuvants, including complete Freund's adjuvant (CFA), alum and plasmid DNA, do not require intact TLR signalling to activate innate immunity [44–46], highlighting the importance of these non-TLR PRRs.

The NLRs encompass a number of sensors that recognize intracellularly encountered PAMPs, and play a critical role in inflammation. They are considered the 'cytoplasmic counterparts of TLRs', and have been demonstrated to recognize several bacterial molecules as well as other danger signals [18]. NOD1 and NOD2 recognize bacterial peptidoglycan (PGN) components [17,18], and activation of NOD1 by PGN has been shown to contribute to antigen-specific $CD4^+$ T cell responses [45]. Nalp3 is important in recognition of the adjuvant alum [44] and can also sense uric acid crystals, an endogenous danger signal [18]. Other NLRs have been implicated in sensing flagellin, accounting for their ability to recognize several live intracellular bacteria [18]. Because TLR-5 also recognizes flagellin it remains unclear how the adjuvant properties of flagellin are mediated by these different receptors [17], although its ability to potentially trigger multiple innate immune pathways may be key to its immunogenicity.

Two RNA helicases, RIG-I and MDA5, are innate cytoplasmic sensors that can detect viral double-stranded RNA. While MDA5 is critical for detection of picornaviruses, RIG-I can recognize influenza virus and Japanese encephalitis virus, displaying differential sensing of RNA viruses. Furthermore, MDA-5, but not RIG-I, recognizes poly I:C and can initiate innate immune responses characterized by type I IFN production and DC activation [47,48]. This provides a redundant innate immune pathway for poly I:C and potentially other double-stranded RNA adjuvants that signal through TLR-3.

Most recently, a cytosolic receptor named DNA-dependent activator of IFN regulatory factors (DAI) was found to recognize plasmid DNA, providing a mechanism for why DNA recognition was not entirely dependent upon TLR-9 signalling. Recognition by DAI occurred in a TLR-independent, CpG motif-independent manner resulting in the production of type I IFNs and chemokines [46]. The identification of non-TLR-dependent innate immune signalling pathways has filled several gaps in understanding the basis for the immunogenicity of many potent adjuvants, and suggests that redundant innate immune signalling pathways may provide synergy and enhance adjuvant function. Further identification of which TLRs and non-TLRs are critical for induction of innate and adaptive immunity during vaccination will prove important for optimizing vaccine design.

Innate immunity and HIV vaccine vectors

Historically, live attenuated vaccines have provided the most effective protection against microbial infections, and generate long-lasting B and T cell responses [1,49]. However, this strategy is not a current option for HIV vaccines because of safety concerns. As discussed earlier, significant efforts are under way to augment the immunogenicity of protein subunit vaccines by developing TLR adjuvants. Additional vaccine strategies include engineering plasmid DNA and live recombinant vectors that express HIV antigens. Several viral and bacterial vectors are being evaluated as potential HIV vaccine candidates [1,49]. Understanding the relationship between innate immunity and the safety and immunogenicity of candidate vaccine vectors is potentially important in determining their utility.

The two viral vector platforms that have advanced the furthest in HIV clinical studies are adenoviruses and poxviruses, because of their favourable safety profile and robust immunogenicity [1,50–53]. Recombinant adenovirus (rAd) vectors have come under intense scrutiny, initially because of their ability to generate potent, durable $CD8^+$ T cell responses, and most recently because of the failure of an rAd5 vector-based HIV vaccine to provide protection in clinical efficacy trials [54]. Several other Ad serotypes (including Ad26, Ad35 and Ad48) have been developed as candidate vectors, as they are biologically distinct from rAd5 and are predicted to be less subject to pre-existing vector immunity compared with rAd5 vectors [55–57]. In previous studies, rAd5 engaged DC and activated innate immunity via TLR-dependent and TLR-independent pathways [58,59]. We have demonstrated further that rAd5 and the rare rAd serotypes, rAd26 and rAd35, signal through a MyD88-dependent pathway that contributed to antigen-specific $CD8^+$ T cell responses in mice. However, only partial MyD88-dependence was observed, suggesting that MyD88-independent pathways are also involved [60]. These observations were consistent with a recent report suggesting the existence of a TLR-independent innate immune sensor for rAds that has not yet been identified [61].

Similarly, poxvirus vectors have been shown to activate both TLR-dependent and TLR-independent innate pathways. Although replicating vaccinia viruses have been used in humans, several safer, less immunogenic poxvirus vectors have been advanced, including modified vaccinia virus Ankara (MVA) [49,62]. Vaccinia virus has been shown to activate innate immunity through a TLR-2-dependent pathway and a TLR-independent, IFN-β-secreting pathway in mice [63]. MVA activated MyD88 signalling, probably via TLR-9 as well as TLR-independent pathways [64]. However, the degree to which TLR and non-TLR innate immune activation contributed to poxvirus-generated adaptive immune responses remains unclear. These novel viral vectors provide tools to evaluate innate immune requirements for generating potent adaptive immunity, as they are generally more

immunogenic compared with protein or plasmid DNA platforms. A deeper understanding of innate immunity triggered by candidate vaccine vectors will probably be critical for improving our understanding of both vector safety and adaptive immunity.

Conclusions

Designing an effective HIV vaccine will require a deeper understanding of the contribution of innate immunity to adaptive immunity and application of this knowledge to the development of improved candidate vaccine vectors and adjuvants. By exploiting TLRs, non-TLRs and DCs, the immunogenicity of protein subunit and plasmid DNA vaccines may be improved substantially [24,38]. Several aspects of adjuvant design deserve further attention, including the development of novel adjuvants targeting TLRs and/or non-TLR PRRs that can be tested with candidate HIV vaccines; additional strategies to target DC and other APCs; and optimized platforms for delivery of antigens and adjuvants to their targets. Moreover, as the expanding array of PRRs, innate immune cell types and downstream signalling pathways indicate, the growing complexity of innate immunity requires ongoing investigation into basic mechanisms addressing how innate immunity is translated to adaptive responses.

Advances in innate immunity have also enabled the 'reverse design' of successful vaccines and adjuvants as historically most vaccines had been formulated empirically. Potent adjuvants such as CFA and bacille–Calmette-Guérin are sensed by multiple TLRs and non-TLRs (NLRs) [17,18]. The live attenuated YF-17D vaccine engages at least four different TLRs and RIG-I [6,7]. These findings support the hypothesis that it may be beneficial for a vaccine vector to utilize multiple redundant or overlapping innate signalling pathways. Continued exploration of how successful vaccines engage innate immunity would provide further insights into vaccine design [4,7,8]. Moreover, by delineating the innate immune pathways utilized by current candidate HIV vectors such as rAd and MVA, the immunogenicity of these vectors could theoretically be designed into new vaccine platforms. Further investigations regarding how viral vectors interact with innate immune pathways will probably be important for advancing the possibility of rational HIV vaccine design.

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